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Evaluation of Chemicals as Inhibitors of Trout Cytochrome P450s[1]

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This study examined the ability of several inhibitors of mammalian cytochrome P450s to affect hepatic P450-mediated monooxygenase activities in microsomes from β-naphthoflavone (BNF)-treated rainbow trout. Three monooxygenase activities, namely, lauric acid (ω-1)-hydroxylase (LA-OH), 7,12-dimethylbenz[*a*]anthracene hydroxylase (DMBA-OH), and progesterone 6β-hydroxylase (PROG-OH) activities were used as functional markers for trout hepatic CYP2K1, CYP1A1, and CYP3A27, respectively. The chemicals that were examined for their inhibitory effects were reversible, quasi-irreversible, or irreversible inhibitors of mammalian P450. At 100 μM concentration, the reversible inhibitors (ketoconazole, miconazole, and clotrimazole) were most potent in inhibiting LA-OH activity. These imidazole compounds, as well as ellipticine, parathion, and α-naphthoflavone, were the strongest inhibitors of DMBA-OH and PROG-OH activities. In addition, isosafrole, piperonyl butoxide, gestodene, 17α-ethynylestradiol, 1-aminobenzotriazole, and 5,8,11,14-eicosatetraynoic acid strongly inhibited PROG-OH activity. The global inhibitors, metyrapone, chloramphenicol, and allylisopropylacetamide, had very little or no inhibitory effect on trout LA-OH and DMBA-OH activities. Triacetyloleandomycin, a CYP3A inhibitor, did not affect PROG-OH activity catalyzed by trout CYP3A27. Diethyldithiocarbamate was an activator of LA-OH activity. None of the above enzyme activities was selectively inhibited by any of the chemicals when used at a concentration of 100 μM. There was no difference in the inhibition of LA-OH activities by representative P450 inhibitors between liver microsomes from untreated trout and BNF-treated trout. The results of this study suggest that inhibition data from mammalian studies could not be directly extrapolated to trout and likely other fish species and that care must be observed when mammalian P450 inhibitors are used to determine the participation of P450s in the metabolism and toxicity of xenobiotics in nonmammalian species. © 1998 Academic Press

The use of chemical inhibitors is one of the common strategies employed in determining whether cytochrome P450s are involved in the hepatic and extrahepatic metabolism of drugs, xenobiotics, and endogenous compounds. Selective chemical inhibitors play an important role especially in elucidating the contribution of a particular cytochrome P450 enzyme in catalyzing the metabolism of drugs and xenobiotics (Halpert *et al.*, 1994; Correia, 1995; Murray and Reidy, 1990). Chemical inhibitors can cause reversible (competitive), quasi-irreversible, or irreversible inhibition of P450s (Ortiz de Montellano and Reich, 1986; Ortiz de Montellano and Correia, 1995). Irreversible inhibitors (suicide inhibitors) are mechanism-based and require metabolism by the target enzyme for them to be converted into intermediates or products that inactivate the enzyme. The requirement for catalysis makes the mechanism-based inhibitors more selective than the reversible inhibitors in their capacity to inhibit a particular P450.

There are a number of compounds frequently employed as inhibitory probes for specific rat and human P450 isoforms. However, some of these diagnostic inhibitors were shown in recent studies to be nonspecific and to inhibit other P450 enzymes. For example, α-naphthoflavone (ANF), which is often used to inhibit CYP1A1 and CYP1A2, was subsequently found to be a potent inhibitor of CYP2C8 and CYP2C9 (Chang *et al.*, 1994). Diethyldithiocarbamate, a effective inhibitor of human CYP2E1 (Guengerich *et al.*, 1991), was later shown to inhibit other cDNA-expressed human P450s such as CYPs 1A1, 1A2, 2A6, 2B6, 2C8, 3A3, and 3A4 (Chang *et al.*, 1994). Methylpyrazole, a powerful inhibitor for human CYP2E1 (Guengerich *et al.*, 1991), could also inhibit CYP2D6-catalyzed bufuralol hydroxylation (Newton *et al.*, 1995). Methoxsalen, a selective inhibitor of human CYP2A6, was subsequently found to inhibit human CYP1A2 (Ono *et al.*, 1996).

Chemical inhibitors may also be useful in identifying the individual P450 enzymes responsible for the metabolism of xenobiotics and endogenous lipophilic compounds in nonmammalian species such as fish. Several inhibitors of mammalian P450s have been employed to inhibit fish P450s (Stegeman and Hahn, 1994). Ellipticine is a strong inhibitor of benzo[*a*]pyrene hydroxylase activity of liver microsomes from flounder (*Platichthys flesus*) (Lemaire and Livingstone, 1994). Aminoanthracene has been proposed as a mechanism-based inactivator of CYP1A in channel catfish (Watson *et al.*, 1995), but its selectivity as a P450 inhibitor is not known. Recent

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studies by Takahashi *et al.* (1995) have revealed that ANF, a known inhibitor of fish CYP1A1, also inhibits the bioactivation of AFB1 and the (ω-1) hydroxylation of lauric acid by liver microsomes from rainbow trout. These reactions are catalyzed by trout CYP2K1, also known as P450 LM2 or LMC2 (Buhler *et al.*, 1994; Miranda *et al.*, 1989; Williams and Buhler, 1983; Williams *et al.*, 1984). Metyrapone, a nonspecific inhibitor of mammalian P450, inhibits benzphetamine *N*-demethylase, ethoxycoumarin *O*-deethylase, and aniline hydroxylase activities in the little skate, *Raja erinacea* (Bend *et al.*, 1973), but not the hydroxylation of lauric acid catalyzed by trout CYP2K1 (Williams *et al.*, 1984). These findings indicate the need for a systematic evaluation of chemical inhibitors of mammalian P450s as diagnostic probes for fish P450s.

The objective of this work was to determine the relative capacity of different inhibitors of mammalian P450 to inhibit P450-mediated monooxygenase activities of rainbow trout liver microsomes *in vitro*. The inhibitory effects of various chemicals on trout P450s were evaluated by studying the effects of these chemicals on selected monooxygenase activities of liver microsomes from β-naphthoflavone (BNF)-treated trout. Liver microsomes from BNF-treated trout were chosen to allow examination of the inhibitory effect of chemicals on both the inducible CYP1A1 form as well as two constitutive P450s, CYP2K1 and CYP3A27. These microsomal preparations were found in preliminary studies to have elevated levels of CYP1A1 with no appreciable changes in CYP2K1 or CYP3A27 expression or enzyme activities. Comparative inhibition studies were also performed on liver microsomes from untreated and BNF-treated rainbow trout to demonstrate that the inhibitory effects of the test chemicals was not influenced by prior treatment of the trout with BNF.

Trout hepatic CYP1A1, previously designated P450 LM4b, was initially purified and characterized by Williams and Buhler (1984) and subsequently cloned by Heilmann *et al.* (1988). The two additional trout P450s, initially called P450s LMC2 and LMC5, were isolated and characterized by Miranda *et al.* (1989) and subsequently cloned, sequenced, and designated as CYP2K1 (Buhler *et al.*, 1994) and CYP3A27 (Lee *et al.*, 1997), respectively. Marker enzyme activities used in this study were lauric acid (ω-1)-hydroxylase (LA-OH), dimethylbenz[*a*]anthracene hydroxylase (DMBA-OH), and progesterone 6β-hydroxylase (PROG-OH) and are catalyzed by trout CYP2K1 (Buhler *et al.*, 1997; Williams *et al.*, 1984), CYP1A1 (Miranda *et al.*, 1997), and CYP3A27 (Miranda *et al.*, 1991), respectively. The use of three different monooxygenase activities made it possible to determine which of the known inhibitors of mammalian P450s are suitable inhibitors of these specific trout P450s.

MATERIALS AND METHODS

Chemicals. The following chemicals were obtained from the sources indicated: miconazole (MC), clotrimazole (CM), metronidazole (MD), norethindrone (17α-hydroxy-19-norpregn-4-en-20-yn-3-one), 17α-ethynylestradiol (EE), sulfaphenazole (SF), diethyldithiocarbamate (DDC), chloramphenicol, cimetidine (CT), metyrapone (MT), triacetyloleandomycin (TAO), proadifen (SKF-525A), ANF, BNF, 4-methylpyrazole (MP), 1-aminobenzotriazole (ABT), octadecynoic acid, spironolactone, and 5,8,11,14-eicosatetraynoic acid (HClEA) (Sigma Chemical Co., St. Louis, MO); 1-benzylimidazole (BI), quinidine (QD), ellipticine (ET), and resorufin (Aldrich Chemical Co., Milwaukee, WI); ketoconazole (KC) and isosafrole (IS) (ICN, Costa Mesa, CA); allylisopropylacetamide (AIA) (Hoffman-La Roche Inc., Nutley, NJ); parathion (PT) (City Chemical Co., New York, NY); piperonyl butoxide (PB) (Pfaltz Bauer, Inc., Stanford, CT); gestodene (GS) (Dr. F. P. Guengerich, Vanderbilt University); [^{14}C]lauric acid (Amersham, Arlington Heights, IL); [^{14}C]progesterone (DuPont NEN, Boston, MA); and [^{3}H]dimethylbenz[*a*]anthracene or DMBA (NCI Radiochemical Repository, Chemsyn Science Laboratory, Lenexa, KS).

Liver microsomes. Microsomes were prepared as described (Williams and Buhler, 1984) from pooled livers of 50 juvenile rainbow trout (*Oncorhynchus mykiss*) that had been untreated or injected with BNF (100 mg/kg ip) 2 days before termination. The washed microsomes, resuspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol and 1 mM EDTA, were frozen at −80°C before use. Protein and P450 contents of liver microsomes were estimated by the methods of Lowry *et al.* (1951) and Omura and Sato (1964), respectively.

Enzyme assays. LA-OH and PROG-OH activities of trout liver microsomes were determined by the method of Giera and van Lier (1991) and Miranda *et al.* (1991), respectively. DMBA-OH activity was determined by the method of DePierre *et al.* (1978) and Nesnow *et al.* (1977). LA-OH, PROG-OH, and DMBA-OH activities of trout liver microsomes were assayed as markers for CYP2K1 (Buhler *et al.*, 1997; Williams *et al.*, 1984), CYP3A27 (Miranda *et al.*, 1991), and CYP1A1 (Henderson *et al.*, 1992; Miranda *et al.*, 1997). A typical incubation mixture consisted of 0.2–0.4 mg protein, 50 mM Tris–HCl, pH 7.4, 1 mM magnesium chloride, 5 mM potassium chloride, 1.0 mM NADPH, substrate, and inhibitor (dissolved in ethanol). The substrate concentrations were 100 μM [^{14}C]lauric acid, 100 μM [^{14}C]progesterone, or 50 μM [^{3}H]DMBA. At these substrate concentrations, the reaction rates were linear for at least 60 min. The final concentration of inhibitor in the incubation mixture was 100 μM. The liver microsomes were preincubated with the inhibitor or ethanol (vehicle, as control) for 30 min at 30°C prior to the addition of substrates (LA, DMBA, or PROG). Incubations were carried out at 30°C for an additional 30 min following the addition of substrate. The major polar metabolites formed during incubation with the radioactive substrates were verified by HPLC.

HPLC analysis. [^{14}C]Lauric acid metabolites were analyzed by the method of Lemaire *et al.* (1992) as modified by Buhler *et al.* (1997) using a Zorbax ODS column (4.6 × 250 mm) at a flow rate of 1.5 ml/min. Metabolites were eluted with acetonitrile (CH_3CN) and water both containing 0.2% acetic acid. After 35 min at 25% CH_3CN, the CH_3CN was increased to 80% in 2 min. At 45 min, the CH_3CN was returned to 25% in 2 min, and the column was equilibrated 23 min before the next injection. Radiolabeled peaks were detected using a Radiomatic A-100 flow monitor. Metabolites were collected, methylated with diazomethane, derivatized with BSTFA [*N,O-bis*(trimethylsilyl)trifluoroacetamide], and then identified by GC-MS (Buhler *et al.*, 1997).

[^{3}H]DMBA metabolites were also analyzed using the same Zorbax column used in the LA-OH assay but with methanol and water as eluants at flow rate of 0.8 ml/min (Chou and Yang, 1979). The methanol was held at 50% for 10 min and then increased with a linear gradient to 100% over 40 min. At 60 min the methanol was returned to 50% in 2 min and the column was equilibrated another 18 min before the next injection. Monitoring was with UV detection at 254 nm and radioisotope detection using the Radiomatic A-100 flow monitor. The metabolites were identified by GC-MS or coelution with authentic standards (Miranda *et al.*, 1996).

[^{14}C]Progesterone metabolites were analyzed using the same Zorbax column as described above with a flow rate of 0.8 ml/min. Solvents used were 60% water and 40% tetrahydrofuran:CH_3CN:methanol (44:29:27, v/v). Detection of

metabolites was as noted for DMBA and the major metabolite, 6β-hydroxyprogesterone, was identified by coelution with an authentic standard and verified by mass spectrometry.

Formation of metabolic-intermediate (MI) complex. The formation of MI complex between the ferrous form of P450 and inhibitor (isosafrole, piperonyl butoxide, TAO, or SKF-525A) was analyzed according to the method of Mansuy *et al.* (1978). The sample and reference cuvettes contained 1 ml each of 0.8–2.0 mg microsomal protein, 0.1 M Tris–HCl, 1 mM magnesium chloride, 0.1 mM EDTA, and 1.0 mM NADPH. The reaction was started by the addition of inhibitor (100 μM) to the sample cuvette. MI complex formation was monitored by repetitive scanning of the difference spectra from 500 to 400 nm over a 30-min period at 30°C. The increase in absorbance at 455 nm relative to 490 nm indicates the formation of the MI complex between P450 and a metabolite of the inhibitor (Franklin, 1976).

Analysis of data. The microsomal monooxygenase activities in the presence of inhibitors were expressed as a percentage of the corresponding control values (incubations containing ethanol). The relative potencies of the different inhibitors tested were then designated as inactive or negligible (≥80% of control values), low (60 to 79% of control values), moderate (40–59% of control values), high (20 to 39% of control values), or very high (0 to 19% of control values). Typically, in the absence of inhibitor (controls with ethanol alone), the lauric acid (ω-1)-hydroxylase, progesterone 6β-hydroxylase, and DMBA hydroxylase activities of liver microsomes from BNF-treated trout were 285, 102, and 335 pmol/min/mg protein, respectively. The results of the inhibition study were obtained from triplicate or quadruplet experiments. A Student's *t* test was used to identify significant differences between control (ethanol) and treated groups, with $p < 0.05$ as the level of significance.

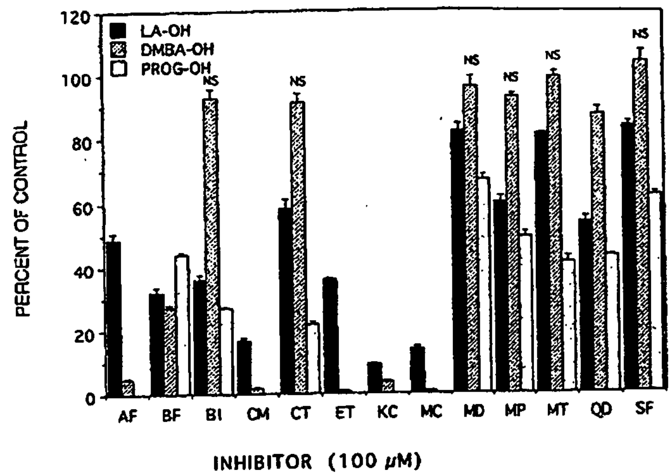

FIG. 1. Inhibition of lauric acid (ω-1)-hydroxylase (LA-OH), DMBA (DMBA-OH), and progesterone 6β-hydroxylase (PROG-OH) activities of trout liver microsomes by reversible inhibitors of mammalian P450. The inhibitors are: α-naphthoflavone (AF); β-naphthoflavone (BF); 1-benzylimidazole (BI); clotrimazole (CM); cimetidine (CT); ellipticine (ET): ketoconazole (KC); miconazole (MC); metronidazole (MD); 4-methylpyrazole (MP); metyrapone (MT); quinidine (QD); and sulfaphenazole (SF). Values are the means ± SE of three to four determinations. Except when indicated by NS (not significant) above the error bars, differences between control and treated groups are statistically significant, $p < 0.05$.

RESULTS

The chemicals evaluated for their ability to inhibit the hepatic microsomal monooxygenase activities of BNF-treated trout belong to three classes of mammalian P450 inhibitors described by Ortiz de Montellano and Reich (1986): (1) reversible inhibitors, (2) quasi-irreversible inhibitors, and (3) irreversible (suicide) inhibitors. The reversible inhibitors selected for this study bind reversibly to the active site and/or ferric P450 heme. The effects of these reversible inhibitors (all at 100 μM) on trout P450 monooxygenases are shown in Fig. 1. ANF inhibited all three monooxygenase activities but PROG-OH was inhibited the most. In contrast, BNF caused greater inhibition of DMBA-OH and LA-OH than PROG-OH. BI had a high inhibitory effect on both LA-OH and PROG-OH activities but not on DMBA-OH activity. CM had a very high inhibitory effect on LA-OH, PROG-OH, and DMBA-OH activities. CT had a negligible inhibitory effect of DMBA-OH but was a modest inhibitor of LA-OH and PROG-OH activities. ET showed a very high inhibitory effect toward DMBA-OH and PROG-OH. KC and MC, like CM, had very high inhibitory effects toward all three P450-mediated activities. However, MD, also an azole compound, had a very low or negligible inhibitory effect on these monooxygenases. MP was inactive toward DMBA-OH but had moderate inhibitory activity toward LA-OH or PROG-OH. MT, which had a negligible inhibitory effect on LA-OH and DMBA-OH activities, was moderately active in inhibiting PROG-OH activity. QD was a moderate inhibitor of LA-OH and PROG-OH with negligible activity toward DMBA-OH. SF was inactive toward DMBA-OH but slightly inhibited LA-OH and PROG-OH activities.

The quasi-irreversible inhibitors of mammalian P450 that were used in this study were methylenedioxyphenyl compounds (piperonyl butoxide and isosafrole) and amines (SKF-525A and TAO). The metabolic intermediates (carbene or nitroso derivatives) of these compounds bind tightly to the ferrous iron of P450 heme to form the MI complex (Ortiz de Montellano and Correia, 1995). The effects of these inhibitors on trout liver monooxygenases are shown in Fig. 2. Isosafrole and piperonyl butoxide were strong inhibitors of PROG-OH but had much lower inhibitory activity toward LA-OH and DMBA-OH. SKF-525A slightly inhibited DMBA-OH but was a modest inhibitor of LA-OH and PROG-OH. TAO, a potent inhibitor of CYP3A4 enzymes in rats and humans, had negligible or no inhibitory effect on the three trout microsomal monooxygenases.

The effects of irreversible inhibitors whose reactive intermediates covalently bind to P450 heme (Ortiz de Montellano and Correia, 1995) on trout microsomal monooxygenases are shown in Fig. 3. GS and EE were most active in inhibiting PROG-OH activity but were poor inhibitors of DMBA-OH activity. AIA had moderate inhibitory effect on PROG-OH but had little or no inhibitory activity toward LA-OH and DMBA-OH. ABT was slightly inhibitory toward DMBA-OH but was highly inhibitory toward PROG-OH. Norethindrone (17α-ethynyltestosterone) was not as active in inhibiting PROG-OH as the other two acetylenic steroids, GS and EE.

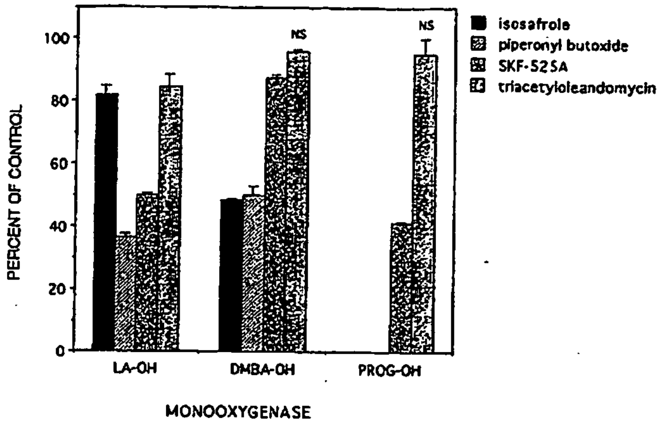

FIG. 2. Inhibition of LA-OH, DMBA-OH, and PROG-OH hydroxylase activities of trout liver microsomes by compounds whose metabolites bind to iron of prosthetic heme of mammalian P450. Values are the means ± SE of three to four determinations. Except when indicated by NS (not significant) above the error bars, differences between control and treated groups are statistically significant, $p < 0.05$.

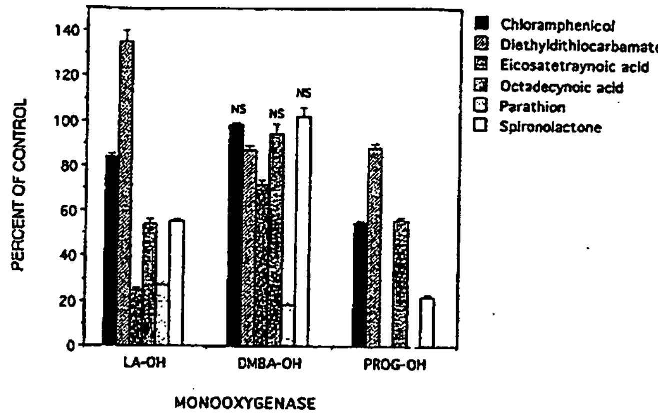

FIG. 4. Inhibition of LA-OH, DMBA-OH, and PROG-OH hydroxylase activities of trout liver microsomes by compounds whose metabolites bind to apoprotein of mammalian P450 or by a chemical (spironolactone) that causes destruction of heme to products that bind to apoprotein of P450. Values are the means ± SE of three to four determinations. Except when indicated by NS (not significant) above the error bars, differences between control and treated groups are statistically significant, $p < 0.05$.

The inhibitory effects of chemicals whose reactive intermediates bind to the apoprotein of mammalian P450 are shown in Fig. 4. PT, 17-octadecynoic acid (inhibitor of CYP4A1), and EA, an inhibitor of arachidonic metabolism in mammals (Ribeiro *et al.*, 1994) were modest inhibitors of lauric acid (ω-1)-hydroxylase activity. Chloramphenicol, a potent inhibitor of CYP2B1 in mammals (Halpert, 1995), was inactive toward DMBA-OH and slightly inhibited LA-OH activity. EA was a potent inhibitor of PROG-OH activity, with low activity toward DMBA-OH. DDC appeared to activate rather than inhibit LA-OH activity of trout liver microsomes. Spironolactone, an agent that irreversibly destroys the heme (Ortiz de Montellano and Reich, 1986), was inhibitory toward LA-OH and PROG-OH but not toward DMBA-OH.

There was a possibility that the inhibition of P450-mediated activities in liver microsomes from BNF-treated trout may be different from that of liver microsomes from untreated trout. However, as shown in Fig. 5, no significant difference was observed in the inhibition of LA-OH activity by EE, KC, MC, PT, or PB in liver microsomes from untreated trout vs inhibition in liver microsomes from BNF-treated trout.

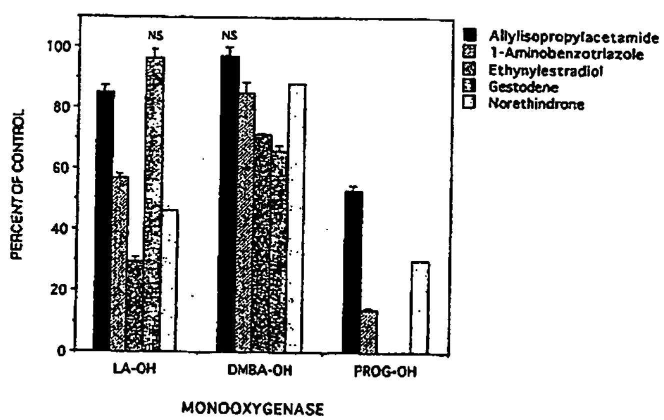

FIG. 3. Inhibition of LA-OH, DMBA-OH, and PROG-OH hydroxylase activities of trout liver microsomes by compounds whose metabolites bind to pyrrole nitrogen of mammalian P450 heme. Values are the means ± SE of three to four determinations. Except when indicated by NS (not significant) above the error bars, differences between control and treated groups are statistically significant, $p < 0.05$.

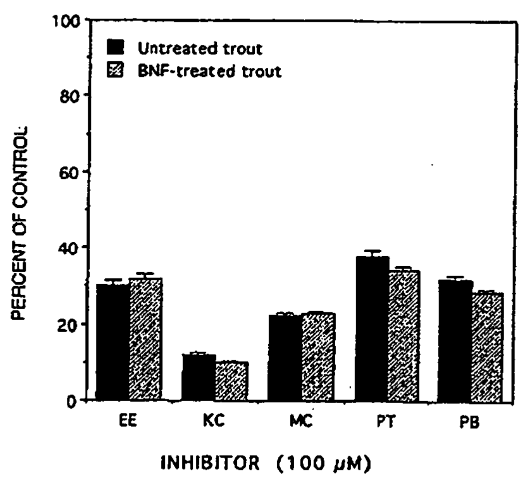

FIG. 5. Inhibition of lauric acid (ω-1) hydroxylase activity of liver microsomes from untreated and BNF-treated rainbow trout. The inhibitors are ethynylestradiol (EE), ketoconazole (KC), miconazole (MC), parathion (PT), and piperonyl butoxide (PB). Values are the means ± SE of four determinations. All values are significantly different ($p < 0.05$, by Student's t test) from the control group. Differences in the means between untreated trout and BNF-treated trout for each inhibitor were not statistically significant.

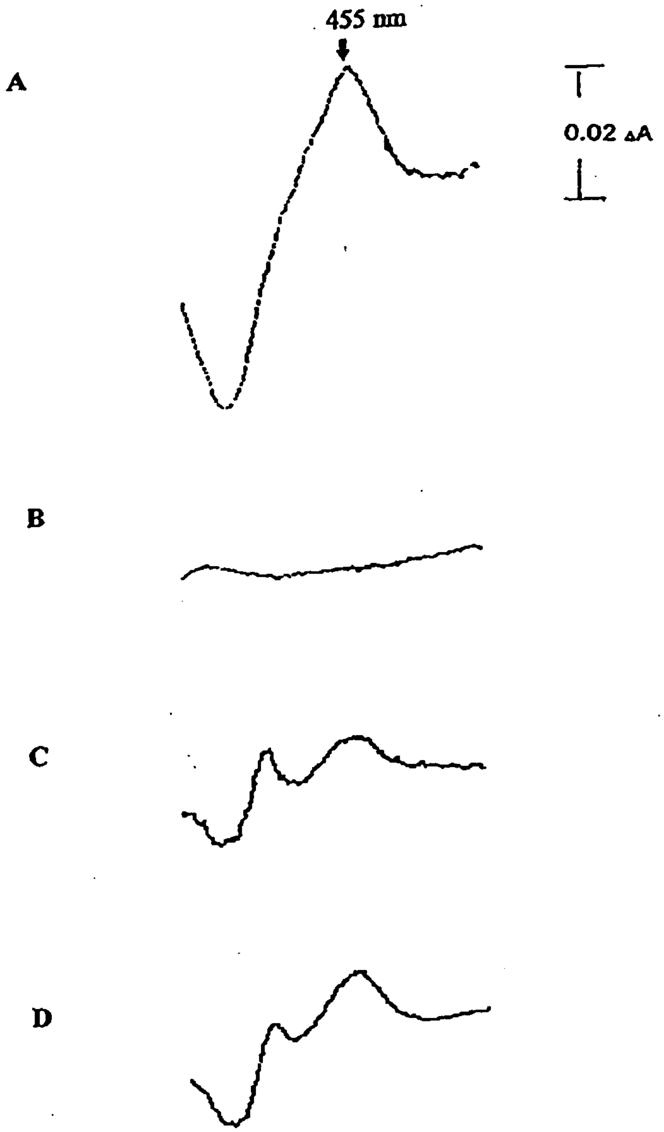

FIG. 6. Formation of complexes of cytochrome P450 from the liver of dexamethasone (50 mg/kg ip)-treated Sprague–Dawley rat with a metabolite of TAO (A) or of cytochrome P450 from the liver of BNF-treated trout with TAO (B), isosafrole (C), and piperonyl butoxide (D) metabolites. Sample and reference cuvettes contained 1 ml each of Tris buffer containing 0.8 mg (rat microsomes) or 2 mg (trout microsomes) protein and 1 mM NADPH as described in Materials and Methods. The reaction was started by the addition of inhibitor (100 μM) to the sample cuvette and the difference spectrum from 500 to 400 nm was recorded after a 30-min incubation period at 30°C. MI complex formation is indicated by the peak appearing at 455 nm.

The lack of inhibitory effect of TAO on trout monooxygenases was intriguing. To help explain this unusual finding, the ability of TAO to form MI complex with trout microsomal cytochrome P450 was examined. As shown in Fig. 6, MI complex formation was observed with TAO and dexamethasone-induced rat liver microsomes but not with liver microsomes from BNF-treated trout. In addition, no MI complex was formed between trout liver microsomes and SKF-525A (data not shown). However, trout liver microsomes incubated with isosafrole or piperonyl butoxide produced a MI complex as shown by the appearance of a 455-nm peak in the presence of NADPH (Fig. 6).

DISCUSSION

This work was conducted primarily to identify chemicals known to inhibit mammalian P450s that can be used as inhibitory probes for trout cytochrome P450s. The trout P450s examined were CYP2K1, CYP1A1, and CYP3A27, which catalyze, respectively, the hydroxylation of LA at the (ω-1) position (Buhler *et al.*, 1997; Williams *et al.*, 1984), the oxidation of DMBA (Henderson *et al.*, 1992; Miranda *et al.*, 1997), and the hydroxylation of PROG-OH in the 6β-position (Miranda *et al.*, 1991). All experiments involved the preincubation of liver microsomes with the inhibiting chemicals since a much greater effect on enzyme activity is observed with mechanism-based inhibitors (Butler and Murray, 1993; Ortiz de Montellano and Correia, 1995). Preincubation was also performed for reversible inhibitors to examine the possibility that these inhibitors may be metabolized by trout liver microsomal enzymes to products that are more inhibitory (or less inhibitory) than the parent compound. Because of the large number of chemicals tested, it was not possible to conduct extensive mechanistic studies to explain the inhibition or lack of inhibition observed for each chemical. However, attempts were made to explain some of the unique observations that were made in this study.

Competitive (reversible) inhibitors such as the imidazole antimycotics (ketoconazole, miconazole, and clotrimazole) were most effective in inhibiting CYP2K1 and were also strong inhibitors of CYP1A1 and CYP3A27. However, the imidazole compound, 1-benzylimidazole, did not affect CYP1A1 catalytic activity, which is consistent with the previous finding that this compound does not inhibit hepatic aryl hydrocarbon hydroxylase activity in 3-methylcholanthrene-treated marine fish, *Archosargus probatocephalus* (Little *et al.*, 1981). The strong inhibitory action of ketoconazole and related compounds is probably explained by their ability to bind to both the lipophilic domain and the prosthetic heme of P450 (Ortiz de Montellano and Reich, 1986).

The two quasi-irreversible inhibitors of mammalian P450, isosafrole and piperonyl butoxide, were inhibitory toward CYP1A1 and CYP3A27 (Fig. 2). However, piperonyl butoxide was more inhibitory than isosafrole toward CYP2K1. In mammals, the inhibitory activity of these compounds depends upon the formation of a MI complex with P450 (Franklin, 1976). However, isosafrole can also inhibit P450 independent of MI complex formation (Murray and Reidy, 1989). We found that both isosafrole and piperonyl butoxide form a MI complex with P450 in BNF-induced trout liver microsomes (Fig. 6). It is possible that all three trout P450s could form an MI complex

with piperonyl butoxide. However, CYP2K1 may not form a MI complex with isosafrole because the latter had very low inhibitory effect toward LA-OH activity, which could be competitive in nature. Further work using purified trout P450s is needed to confirm this hypothesis.

TAO, a tertiary amine and a quasi-irreversible inhibitor of trout P450, is a selective inhibitor of mammalian CYP3A enzymes through formation of a MI complex (Franklin, 1991; Murray and Reidy, 1990; Roos *et al.*, 1993). The MI complex, formed between the ferrous form of P450 heme and a nitroso intermediate of TAO, has a λ_{max} of 455 nm similar to the MI complex between an isosafrole metabolite and P450. MI complex formation results in noncompetitive inhibition of substrate oxidation. In the present study, TAO slightly inhibited CYP2K1-mediated LA-OH activity but did not inhibit CYP3A27-mediated PROG-OH activity. The lack of inhibitory effect of TAO on CYP3A27 may be explained by the inability of this enzyme to form a MI complex with TAO (Fig. 6). CYP3A27 may be inactive in catalyzing TAO *N*-demethylation, an obligatory step in the formation of the nitroso intermediate, which binds to the P450. In contrast, SKF-525A, also an amine and a quasi-irreversible inhibitor of mammalian P450 (Murray, 1988), was found to inhibit CYP3A27 and CYP2K1. However, the inhibition may be competitive since SKF-525A did not form an MI complex with trout P450. Further kinetic studies are needed to verify this assumption.

Acetylenic steroids such as EE, GS, and norethindrone are selective suicide inhibitors of CYP3A4 (Guengerich, 1988, 1990). These compounds are converted to reactive intermediates that alkylate the heme of P450 rendering the enzyme inactive. We found that both gestodene and EE were potent inhibitors of trout CYP3A27-mediated PROG-OH activity but not of LA-OH or DMBA-OH activities. Norethindrone was not as potent as EE or GS in inhibiting CYP3A27, a finding that is consistent with that reported by Guengerich (1990), who demonstrated differential inhibitory effects of acetylenic steroids on CYP3A4. It is possible that both competitive and mechanism-based inactivation of trout P450s may occur with these three acetylenic steroids as in the case of thiosteroids (Underwood *et al.*, 1992) and PT (Murray and Butler, 1995). The competitive inhibition component of these compounds could be reduced or removed by washing the microsomes with serum albumin (Underwood *et al.*, 1992) or diluting the microsomes 20-fold with buffer (Guengerich, 1988) after preincubation and prior to the addition of substrate. As mechanism-based inactivators, GS and EE might be used at lower concentrations as specific inhibitor probes for CYP3A27, but additional mechanistic studies are needed to verify this point.

The suicide inhibitors such as AIA and ABT whose reactive metabolites alkylate the heme of mammalian P450 (Bornheim *et al.*, 1987; Ortiz de Montellano *et al.*, 1981; Halpert *et al.*, 1994) had a slight (AIA) or a strong (ABT) inhibitory effect on CYP3A27. Neither agent inhibited trout CYP1A1. With rat CYP1A1, ABT, but not AIA, was inhibitory (Ortiz de Montellano *et al.*, 1981). Unlike rat CYP1A1, trout CYP1A1 may not be able to metabolize ABT to benzyne, the reactive inhibitory species (Ortiz de Montellano and Correia, 1995).

Mechanism-based inhibitors whose metabolites bind to the protein moiety of P450 have variable effects on trout P450s (Fig. 4). Chloramphenicol, which is a mechanism-based inactivator of CYP2B1 (Halpert *et al.*, 1994), was an inhibitor of CYP3A27-mediated PROG-OH but not of CYP1A1. It is not known at this time if CYP3A27 can convert chloramphenicol to oxamyl chloride, which binds to lysine residues of the P450 protein or if CYP3A27 inhibition by chloramphenicol is simply competitive in nature. DDC, a mechanism-based inactivator of CYP2E1 (Chang *et al.*, 1994), appeared to activate rather than inhibit CYP2K1. This activation of trout P450 by a compound known to inhibit P450 is analogous to the activation of CYP3A by ANF (Shou *et al.*, 1994), an inhibitor of CYP1A1. PT, a sulfur-containing compound like DDC, was observed to be a potent inhibitor of CYP2K1, CYP1A1, and CYP3A27. PT, a competitive and an irreversible inactivator of CYP2B1 (Murray and Butler, 1995), may inhibit trout P450 enzymes in a similar manner.

Certain mechanism-based inhibitors of mammalian P450 can cause inhibition of P450 by two different suicidal mechanisms. For example, the acetylenic fatty acid, 10-undecynoic acid, inactivates rat liver CYP2B1 by heme *N*-alkylation but inactivates CYP4A1 by protein acylation (CaJacob *et al.*, 1988). This acetylenic fatty acid inhibits the ω-hydroxylation of lauric acid (Ortiz de Montellano and Reich, 1984). In the present study, two acetylenic fatty acids, EA and octadecynoic acid, were found to inhibit the LA-OH and PROG-OH activities. It should be noted that ω-hydroxylation of lauric acid is catalyzed by CYP2K1 in trout (Buhler *et al.*, 1998). No ortholog of CYP4A1 has been found in trout upon immunoblotting of trout liver microsomes with anti-rat CYP4A1 antibody kindly provided by Dr. Richard Okita from Washington State University (data not shown). CYP4T1, a new member of family CYP4, has been found in rainbow trout liver (Falckh *et al.*, 1997), but its catalytic activity toward CYP4A1 substrates has not been characterized. The question as to whether EA inhibits CYP3A27 by protein alkylation or heme alkylation was not resolved in this study.

In summary, the results of this study showed which of the reversible and irreversible inhibitors of mammalian P450 are effective inhibitors of trout P450. At 100 μM concentrations, the most potent inhibitors (more than 80% inhibition) of trout P450s were as follows: ketoconazole, miconazole, and clotrimazole for CYP2K1; ellipticine, miconazole, clotrimazole, ketoconazole, ANF, and parathion for CYP1A1; and ellipticine, miconazole, clotrimazole, ketoconazole, ANF, parathion, isosafrole, piperonyl butoxide, gestodene, EE, EA, and ABT for CYP3A27. Unlike in mammals, none of the inhibitors tested were specific inhibitors of trout P450. TAO was inactive toward CYP3A27 and CYP1A1 whereas chloramphenicol and AIA were inactive toward CYP1A1. Other mammalian P450 inhibitors were also inactive

against one or more trout P450s. Thus, caution must be observed in the use of mammalian P450 inhibitors as probes for the involvement of P450 in the metabolism and toxicity of chemicals in trout. The mechanisms by which the mechanism-based inhibitors of mammalian P450 inhibit trout P450s are now being investigated in our laboratory.

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